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13. SUPPLEMENTARY NOTES

14. ABSTRACT : The overall objective of this project is to develop targeted therapy tools directed against the breast cancer vasculature. We aim to bypass many obstacles in the field by developing a treatment based on the targeting of tumor blood vessels with T cells bearing chimeric immune receptors (CIRs). We have constructed and tested first generation CIR lentiviral vectors targeting hTEM1, which were derived from a panel of scFvs of varying affinity. These constructs signal through CD3ζ but can be augmented by the addition of costimulatory molecules (CD28 and 4-1BB). In vitro, only T cells bearing the scFv78 CIR were activated upon co-culture with recombinant hTEM1 protein. However, no activation was observed when scFv78 CIR bearing T cells were co-cultured with cells expressing hTEM1 on the surface. Additional strategies, including hinge elongation and the utilization of loadable CIR bearing T cells, also failed to elicit a response against the hTEM1 bearing target cells. We engineered a new yeast-display scFv library from patients with ovarian cancer to isolate a second generation of anti-TEM1 scFvs. We validated a panel of multivalent antibodies for binding to recombinant proteins by ELISA assays.. We have generated CIRs against PSMA using scFv (PZ1) and T cells bearing PZ1 and CD28 ζ produce a robust polyfunctional repertoire of cytokines. A tumor vascular mouse model expressing human TEM1 was developed and PET imaging with [124I]-MORAb-004 indicated TEM1-specific accumulation of antibody. We have established optimized HSV-tk as extra safety and tracer for PET imaging.

15. SUBJECT TERMS
Chimeric Immune Receptor(CIR), tumor vascular targeting, PET, TEM1, PSMA, scFv

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INTRODUCTION

In spite of great incremental progress, the incidence and m ortality of breast cancer remain high. Molecular targeting is poised to enhance sensitivity and s pecificity of imaging and c an be simultaneously exploited for therapy. Tumor blood vessels are known to be different from vessels of normal tissues(*1-3*) and could offer unique molecular targets for developing molecular imaging or targeted therapeutics(*4, 5*). The Objectives of this proposal are to develop targeted therapy tools directed against the breast cancer vasculature. The central hypothesis that we wish to test is that immune-gene therapy with T-bodies expressing CIR directed against tumor vascular targets and bi- or tripartite signaling modules circumvents many limitations in the field and represents a powerful approach to treat breast cancer. Our specific aims are: 1) Engineer T cells to express T-bodies that effectively target TEM1-positive vasculature using lentiviral transduction of chimeric immunoreceptors (CIR) comprising anti-TEM1 scFv, CD3ζ ,CD28 and/or 4-1BB; 2) Optimize T-bodies for safety by separating the anti-TEM1 CIR carrying CD3ζ (stimulatory) from a CIR recognizing prostate specific membrane antigen (PSMA), which will carry CD28 and/or 4-1BB (costimulatory). 3) Test T-bodies against breast cancer in vivo in orthotopic and metastatic breast cancer in mice.

BODY

Task 1. Engineer T-bodies attacking TEM1-positive endothelial cells.

a. *Develop the first generation CIR lentiviral constructs targeting TEM1 with a panel of scFvs, with CD3 and costimulatory modules (4-1BB and/or CD28)*
b. *Prepare the lentivirus and test in vitro with T cells for targeting TEM1 endothelial cells in 2-D culture by cytokine, proliferation, and cytotoxicity assays (Months 3-6).*
   AND
c. *Define CIR affinity and isolate best for testing in T-bodies; will test avidity and in vitro cytotoxicity*

In the annual report submitted July 2011 we reported that five unique CIRs against hTEM1 were generated using scFv fragments isolated from a human derived yeast-display library. Among all the series of scFv tested, only scFv78 CIR bearing T cells were activated after co-culture with immobilized protein. However, such scFv78 CIR bearing T cells were **not** activated after co-culture with hTEM1 positive target cells. We tested hinges of different length; posited that the CD8α hinge did not sufficiently extend from the T cell surface, limiting the ability of scFv78 to interact with hTEM1 on the surface of the target cell lines. With a more flexible IgG4 hinge elongating the hinge region by 186 amino acids, such CIR bearing T cells incorporating this construct, unfortunately, also failed to activate in the presence of hTEM1 positive target cells.

In order to use anti-TEM1 PET to assist TEM1-specific adoptive therapy, we have engineered de novo TEM1-specific antibodies of scFv78 fused to human IgG1(Fc, CH2, CH3 and hinge). We have found that these multivalent constructs showed higher avidity, longer serum half-life in vitro, better blood pharmacokinetics and ability for direct 124-I labeling when compared to scFv78 we identified preciously(*6*) (Figure 1). We are currently testing their applicability of immuno-PET. We also engineered a new yeast-display scFv library from patients with cancer instead of autoimmune condition (*7*), and a new recombinant TEM1 protein (rTEM1) expressed in mammalian cells instead of bacteria, to isolate a second generation of anti-TEM1 scFvs. These novel anti-TEM1 scFv were validated by ELISA assays for binding to recombinant proteins. However, despite satisfying binding to the recombinant protein rTEMs, we found that, as the first generation, the second generation anti-TEM1 scFv could not detect cell surface expressed TEM1. We changed model system and used different scFv isolated from the same library with the same method (*8*) to understand the limits of our system of selection. We found that while detection assays performed by flow cytometry analysis were not predictive of scFv function as CIRs, capture assays performed by ELISA assays were. We asked for a no-cost extension to retest the second generation of anti-TEM1 scFvs using BBIR transduced T cells ($BBIR^+$T cells). $BBIR^+$T cells recognize and bind exclusively to cancer cells pre-targeted with specific biotinylated molecules (*9*). But again, we were unable to demonstrate specific killing targeted by anti-TEM1 scFv. We concluded that the current methods of scFv screening are not predictive of T cell targeting.

In parallel to hTEM1, we have identified several additional TVMs by quantitative real-time polymerase chain reaction (qRT-PCR) of tumor endothelial cells isolated by laser-capture microdissection(*10*). These TVMs include tumor endothelial marker 5 (TEM5), tumor endothelial marker 7 (TEM7), CUB-domain–containing protein 1 (CDCP1), endothelin receptor B (EDNRB), endocan (ESM-1), and Frizzled-10 (FZD10, or CD350). To generate antibodies against these TVMs, we first expressed the extracellular domain of each target antigens as soluble protein. We designed a panel of novel vectors utilizing various easy-to-express proteins (i.e. SUMO, Fc, the extracellular domain of mTEM7, etc.). Among all the vectors we constructed (Figure 2A), TVMs fused to extracellular domain of mTEM7 yielded the highest expression (Figure 2B). Using these vectors, almost all of the antigens have been successfully produced, including the extracellular domains of hTEM5, mTEM5, hFAP, mCDCP1, hFZD10, mFZD10, hEDNRB, hSP17 and mSP17(Figure2). To isolate high affinity antibodies that target these novel tumor markers, we have constructed a novel yeast display library of human recombinant antibodies derived from B cells of ovarian cancer patients (*7*). Using this library and our previous TTP patient derived scFv library, after several rounds of magnetic sorting, we have isolated antigen binding populations for hTEM1, hTEM5, hTEM7, hCDCP1, hFAP, hFZD10, hEDNRB, hESM-1 and hSPA17. We are currently characterizing the high affinity antibodies we isolated by flow cytometry based cell sorting and make CIRs constructs based on these scFvs to target above tumor vasculature markers.

Task 2. Optimize T-bodies for affinity and for safety

a. Generate T-bodies transduced with hTEM1 CIR carrying signal 1 and PSMA/TEM7R CIR carrying signal 2 (and vice versa), using affinity-optimized scFv.
b. Develop endothelial cells expressing hTEM1 only, PSMA only or both ligands for in vitro and vivo experiments.
And
c. prepare the lentivirus and test in vitro with T cells for targeting TEM1 endothelial cells in 2-D and 3-D culture by cytokine, proliferation, and cytotoxicity assays.

PSMA has been reported to express in breast carcimoma (*11*). Immunohistochemistry was performed on a tissue microarray containing 13 primary ovarian cancer specimens. Over half of the samples expressed PSMA on the tumor vessels (Figure 3). Currently, the array is being analyzed to determine the percentage of positive vessels from each tumor core, and also the intensity of staining. Flow cytometry was also used to confirm the expression of PSMA on patient derived tumor endothelial cells. In previous annual report we constructed CIRs against PSMA using the PZ1 scFv. Among the series of lentiviral CIR vectors we constructed, which confer signaling through CD3ζ, CD28ζ, 4-1BBζ, or CD28BBζ. T cells bearing the PZ1 CIR with CD28ζ signaling produce the most robust polyfunctional repertoire of pro-inflammatory cytokines. PSMA isoform 1 is the most abundant PSMA isoform detected in prostate cancer, whereas PSMA isoform 2 has greater prevalence in benign tissue. For this reason, we hypothesize that PSMA isoform 1 may be physiologically relevant in the tumor neovasculature as well. We have characterized the ability of the Pz1 chimeric antigen receptor to specifically recognize PSMA the membrane bound and the cytosolic isoform. Immortalized human microvascular endothelial cells, HMEC-1, were transduced to express either isoform 1 or 2. Pz1 CAR bearing T cells were co-cultured with HMEC-1, HMEC-1PSMA1, or HMEC-1PSMA2, and revealed exclusive specificity for the HMEC-1 cells that expressed PSMA isoform 1. This restriction in specificity allows CIR bearing T cells to distinguish between the two prevalent membrane isoforms of PSMA, and highlights a potential safety feature of the Pz1 scFv should the isoform distribution be unique between the normal and tumor blood vessels.

Task 3. Test T-bodies against breast cancer in vivo.

a. Establish human endothelium xenografts in the NSG(NOD/SCID/γc-/-) mouse model by injection breast cancer cell mixed with immortalized endothelial cells stably expressing TEM1 subcutaneously into the mammary pads (orthotopic) and the flank or the liver (metastatic). Observe in vivo by PET and bioluminescence.

b. Determine whether xenografts harbor functional vasculature from the xenograft by histochemical and immunohistochemical assays.

c. Determine if the function of T-cells tranduced with CIR against TEM1 with Winn assay and after injection intratumorally, intravenously, by tumor volume, bioluminescence, PET and MRI.

A significant hurdle in testing anti-vascular agents is the availability of animal models expressing human vascular targets. In previous annual report. we have developed the first mouse model of tumor endothelium expressing human TEM1 in immunodeficient mice in vivo. In parallel to the development of this xenograft mouse model, we also engineered MS1 cells to express human PSMA and both hTEM1 and PSMA. These models will be used to test the ability of first generation (single CIR) and second generation (split-signal1 and signal 2 CIR) T cells transferred intravenously to reject TEM1+ PSMA+ tumors in vivo.

Using above model, we also established TEM1-specific molecular PET imaging of hTEM1-expressing tumor vasculature with [124I]-MORAb-004. PET images of [124I]-MORAb-004 distribution 18 h following intravenous administration (5 µg/mouse) revealed excellent uptake in mice with tumors harboring hTEM1-positive vasculature, with negligible uptake in tumors whose vasculature did not express hTEM1 or in the blood pool, and abdomen.

A major concern of adoptive T cells therapy is safety. To provide a second layer of safety, we hypothesize that a suicide gene can be used to transduce CIR-bearing T cells. The *herpes simplex* virus-1 thymidine kinase (HSV1-tk) gene is a good candidate since it also enables tracing the T-cells with FIAU/FHBG PET.

We have constructed a panel of lentiviral vectors with different promoters driving HSV-TK and have tested for the level of expression (Figure 4). These experiments will help us make decisions regarding optimization of TK expression. Human primary CD4 T cells were infected with lentivirus coding HSV-TK under different promoters (CMV, EF1a MSCV, and Ubi). Cells were treated with 0.5ug/mL puromycin for 3 days, then analyzed for vitality (7-AAD negative) and expression of GFP-puro. We have found that MSCV promoter is the best candidate as CMV quickly loses its promoter activity. Currently, we are testing the RNA and protein level of HSV-tk in these T-cells and optimizing our protocol for expansion of the puromycine-selected T cells. We plan to evaluate the function of the promoter-optimized HSV-tk T-cells in in vitro and vivo killing-assays using ganciclovir as suicide treatment. We have set up PET with ^{18}F-FHBG and ^{124}I-FIAU, so that we are able to evaluate tracking the location of adoptive T cells in vivo to estimate their quantity in the tumor and follow their fate in time.

KEY RESEARCH ACCOMPLISHMENTS:

- We developed first generation CIR lentiviral vector constructs targeting hTEM1 with a panel of scFvs of different affinity (2nM to 4uM). These constructs containing CD3 AND costimulatory molecules (CD28 and 4-1BB). However, no activation was observed when co-culture was performed with target cells expressing hTEM1 on the surface.
- We engineered a new library of human recombinant antibodies from ovarian cancer patients that was used to screen for additional anti-TEM1 scFv.
- In parallel to hTEM1, we have developed de novo mammalian expression vectors for expressing additional TVMs. These TVMs are purified and used to pan our scFv library.
- We have generated CIRs against PSMA using scFv (PZ1). PZ1 CIR bearing T cells recognize only PSMA isoform 1 but not isoform 2.
- We established a tumor vascular mouse model expressing human TEM1 and PET imaging with [124I]-MORAb-004 indicated TEM1-specific signal accumulation.
- To add extra safety and enable tracing homing of CIR-bearing T cells, we developed HSV-tk lentiviral vectors and optimized the promoter.

REPORTABLE OUTCOMES

1. Manuscripts submitted to *Cancer Research*:
   -Antibody-Based Tumor Vascular Theranostics Targeting the Human Endosialin/TEM1 In Vivo
   Chunsheng Li, Ann-Marie Chacko, Jennifer Swails, Jia Hu, Kosei Hasegawa, Luigi Grasso, Wafik S. El-Deiry, Nicholas Nicolaides, Vladimir R. Muzykantov, Chaitanya R. Divgi, George Coukos
   - Novel human anti-B7-H4 recombinant antibodies overcome B7-H4-mediated T-cell inhibition and potentiate T cell anti-tumor responses. *Denarda Dangaj, Evripidis Lanitis, Aizhi Zhao, Shree Joshi, Yi Cheng, Raphael Sandaltzopoulos, Daniel J. Powell Jr. and Nathalie Scholler*
2. Manuscript in preparation for *Journal of Nuclear Medicine)*

Developing 124-I MO RAb-004 immuno-PET for the Endosialin/TEM1 In Vivo by *Ann-Marie Chacko, Chunsheng Li, Jia Hu, Luigi Grasso, Vladimir R. Muzykantov, Chaitanya R. Divgi, George Coukos*

3. Manuscript in preparation for *Protein Expression and Purification*:
Design, expression, purification of multivalent, high-affinity full human scFv variants against the tumor vascular marker TEM1/CD248 by *Chunsheng Li, Jia Hu, Ann-Marie Chacko, Junying Wang, Aizhi Zhao, George Coukos*

CONCLUSION

To develop targeted therapy tools directed against the breast cancer vasculature, we are posed to apply immune-gene therapy with T-bodies expressing CIR directed against tumor vascular targets and multiple signaling modules to circumvents many limitations in the field. Our specific aims are: 1. Engineer T-bodies effectively targeting TEM1-positive vasculature using lentiviral transduction of chimeric immunoreceptor (CIR) comprising anti-TEM1 scFv, CD3ζ ,CD28 and/or 4-1BB; 2. Optimize T-bodies for safety by separating anti-TEM1 CIR carrying CD3ζ (signal 1) CIR recognizing prostate specific membrane antigen (PSMA) carrying CD28 and/or 4-1BB (costimulatory signal 2). 3. Test T-bodies against breast cancer in vivo in orthotopic and metastatic breast cancer in mice.

We have constructed and tested the first generation CIR lentiviral vectors targeting hTEM1 with a panel of scFvs of different affinity. These constructs containing CD3 AND costimulatory molecules (CD28 and 4-1BB). In vitro only T cells bearing scFv78 CIR can be activated upon co-culture with recombinant hTEM1 protein. However, no activation was observed when coculture with cells expressing hTEM1 on the surface, with loaded via a shorter CD8, a longer IgG4 hinge, or Streptavidin-biotin bond. Additional scFv is being screened now for high affinity scFv against TVMs. We have generated CIRs against PSMA with only signal 1 or signal 2 using scFv (PZ1) and T cells bearing PZ1 and CD28 ζ produce robust polyfunctional repertoire of cytokines. A tumor vascular mouse model expressing human TEM1 has been developed and PET imaging with [124I]-MORAb-004 indicated TEM1-specific accumulation of antibody. We have established optimized HSV-tk as extra safety and tracer for PET imaging.

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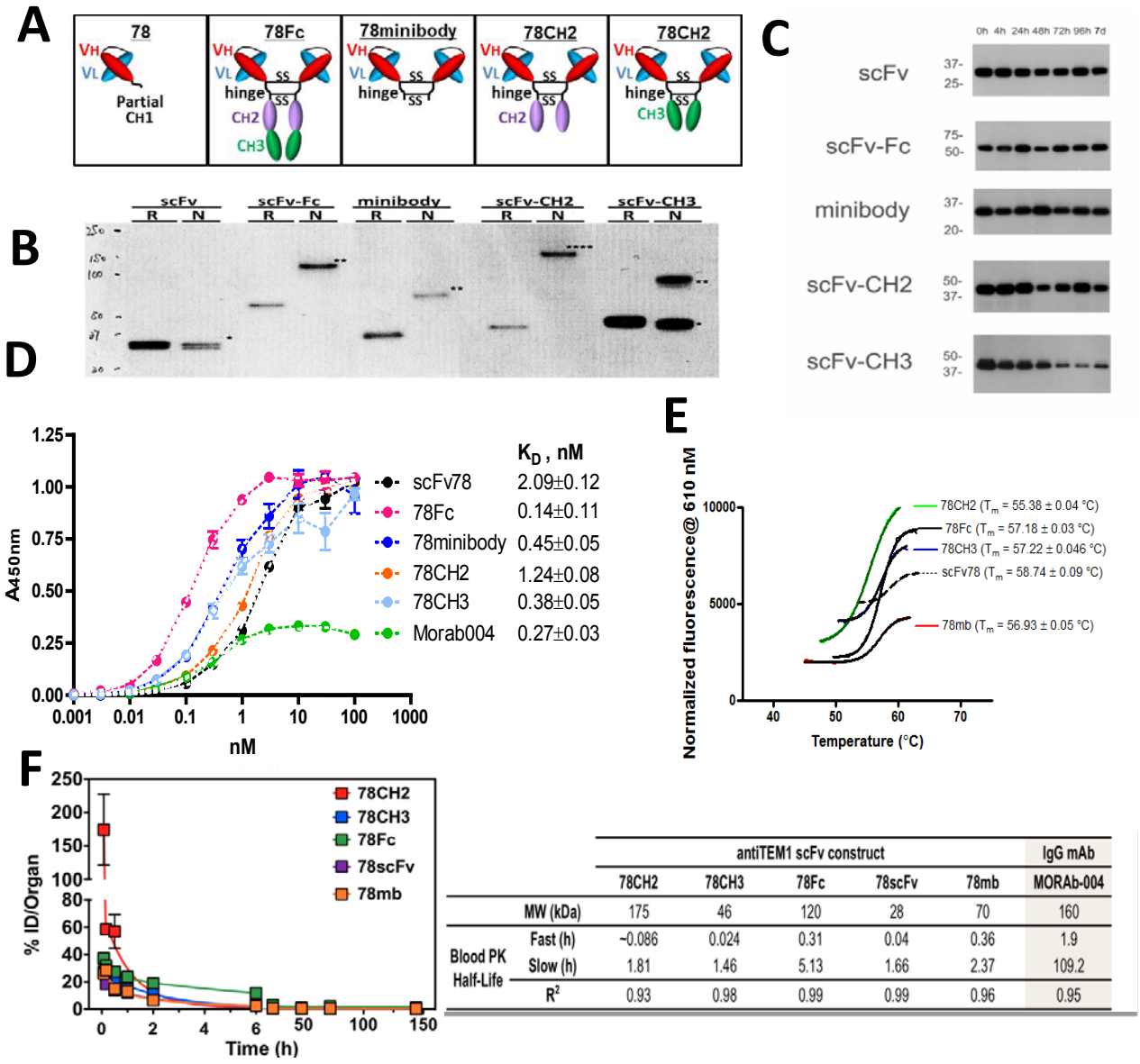
Appendices

NA

Supporting DATA



		antiTEM1 scFv construct					IgG mAb
		78CH2	78CH3	78Fc	78scFv	78mb	MORAb-004
	MW (kDa)	175	46	120	28	70	160
Blood PK Half-Life	Fast (h)	~0.086	0.024	0.31	0.04	0.36	1.9
	Slow (h)	1.81	1.46	5.13	1.66	2.37	109.2
	R^2	0.93	0.98	0.99	0.99	0.96	0.95

Figure 1 (A) Conceptual design for TEM1 antibodies based on scFv78. (B) Multimeric status of constructs by Western blot under reducing (R) and non-reducing (NR) conditions. (**C**)in vitro plasma stability analysis of the constructs, (**D**)KD analysis using Live cell ELISA of scFv-Fc and scFv-CH3 suggesting higher Kd than scFv78 and Morph004.(**E**) Thermal stability analysis of constructs. (**F**), in vivo blood PK study indicated that 78Fc has superior fast and slow half-life compare to scFv78.

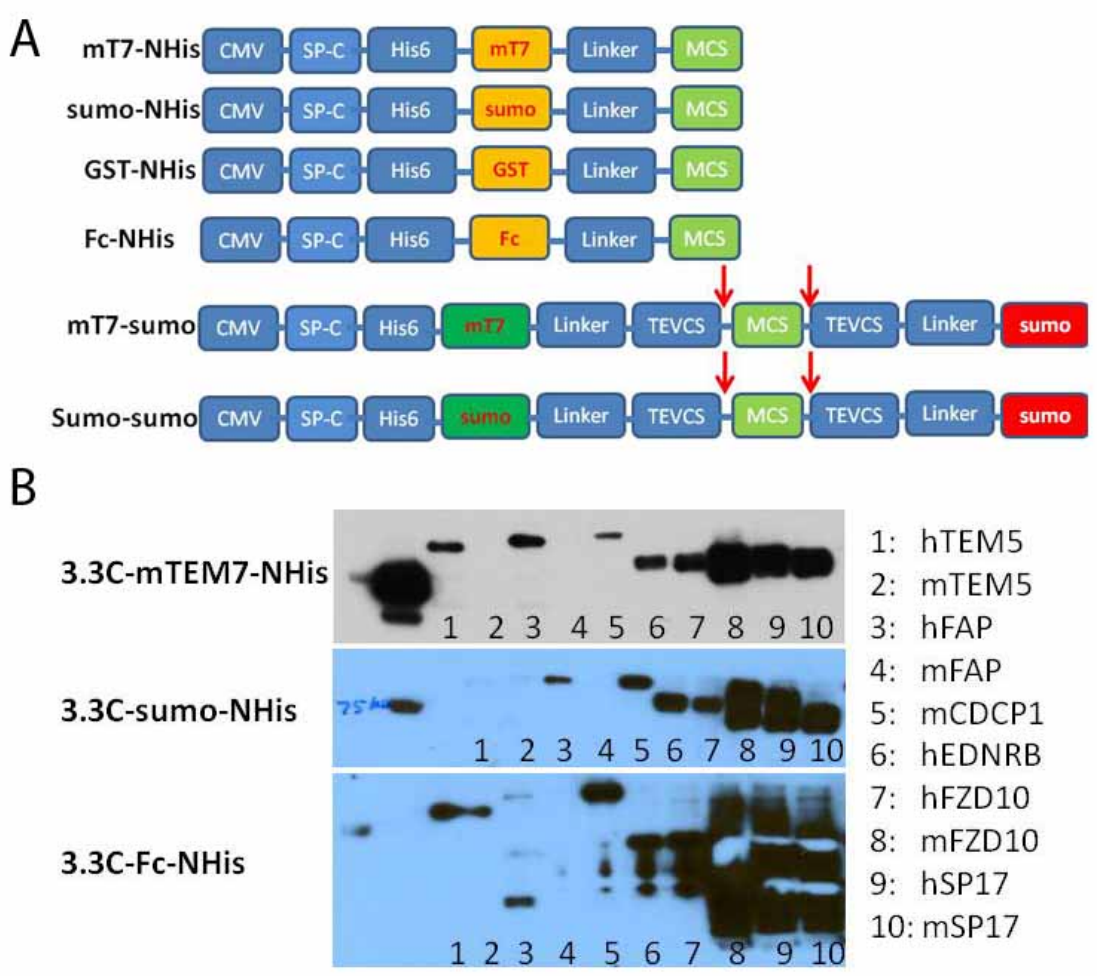


Figure 2. (A) Novel expression vector design for TVMs with difficult expression. (B) Protein expression of TVMs by Western blot.

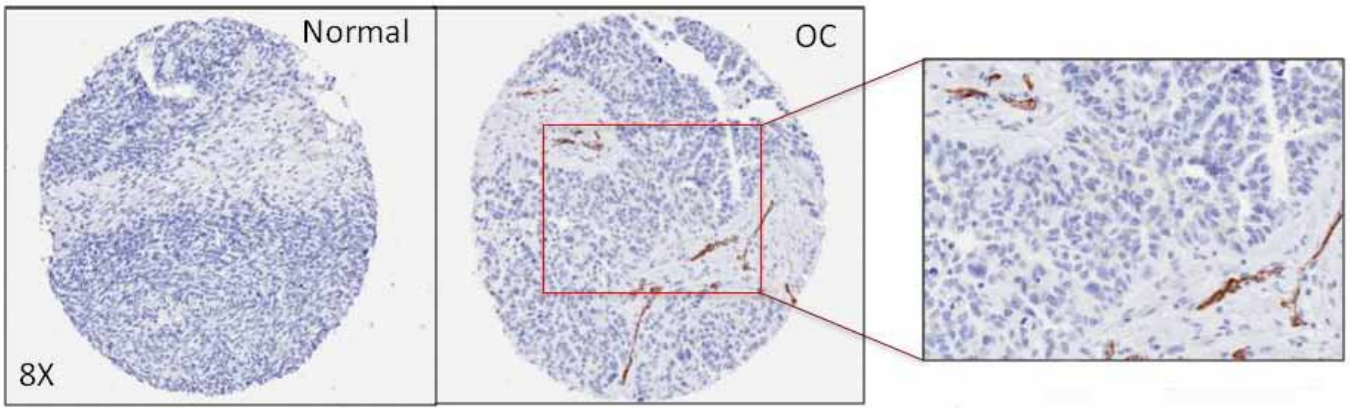


Figure 3. Expression of PSMA in the vasculature of ovarian cancer by immunohistochemistry

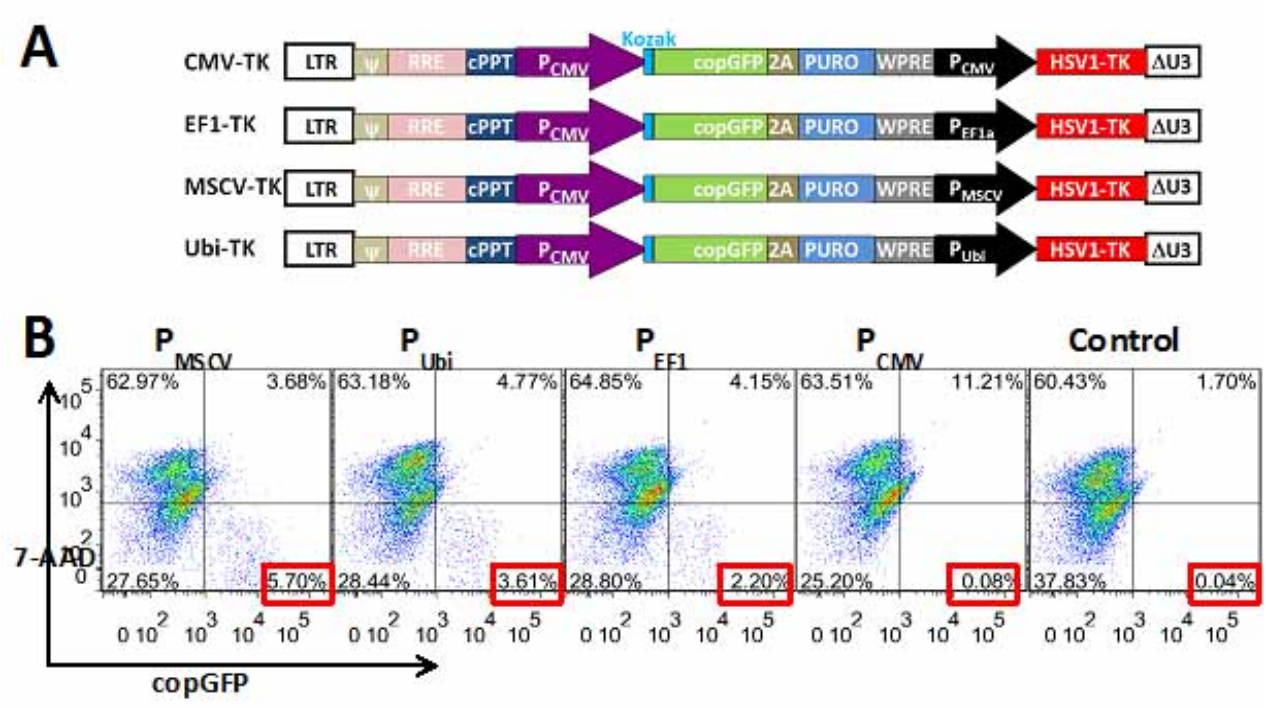


Figure 2.A. Design for optimizing lentiviral vectors with CMV, EF1a, MSCV and Ubi promoters for driving HSV-TK, B. Puromycin selection of infected T cells using HSV-TK antibody